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Spore Fine Structure in *Clostridium cochlearium*

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The fine structure of *Clostridium cochlearium* was examined by use of thin sections, negative stains, and carbon replicas. Particular attention was given to details of the sporulation process and to fine structure of the spores. Spore coat formation was well advanced before the first evidence of cortex formation was noted. Three distinct spore coats were detected, the outermost of which was composed of seven layers. In addition, the spores possessed tubular appendages of variable length attached to one end of the spore. These differed in a number of respects from those described for other clostridia.

Clostridium cochlearium is characterized morphologically in the 7th edition of *Bergey's Manual* as a rod-shaped bacterium which forms ovoid to ellipsoid terminal spores in sporangia distinctly swollen at sporulation (1). Our objective has been to provide an electron microscope characterization of the organism with special emphasis on the sporulation process and spore fine structure. The strain of *C. cochlearium* used in this study exhibits a number of unusual spore structures and features which have not, to the best of our knowledge, been described.

MATERIALS AND METHODS

C. cochlearium 6B. This anaerobic sporeformer was isolated from the bottom sediment of a freshwater pond on The University of Texas campus in Austin, Texas. It was identified as *C. cochlearium* by Louis D S. Smith, Virginia Polytechnic Institute, and, by conventional taxonomic criteria (1), is representative of that species.

Culture methods. The organism was inoculated onto the surface of 2% agar plates of Thioglycollate Medium (Difco) and incubated at 30 C in desiccators with wet oats to provide an anaerobic environment (17). The time course of culture development was as follows: 6 days, sporangia with refractile spores; 8 days, a few free spores; 10 days, 50% free spores; poor synchrony at all stages.

Cell harvest. For carbon replicas, specimens at different stages of development were removed from the culture plates with water, washed six times with demineralized water, and finally resuspended in demineralized water. No particular effort was made to separate cells in different morphological stages. For negative stains and sections, specimens were used directly from the culture plates.

Carbon replicas. Washed cells were placed on freshly cleaved mica squares, shadowed with platinum, and coated with carbon. Further handling of the carbon replica films was by methods described previously (18). Finished replicas were placed on 200-mesh copper grids for examination.

Negative stains. Specimens were negatively stained with uranyl acetate, *p*H 4.3.

Fixation and sectioning. After a preliminary fixation for 1 hr at 4 C in 0.5% glutaraldehyde (19), specimens were subjected to a routine Kellenberger osmium fixation at 4 C for 17 hr (8). Fixed, dehydrated specimens were embedded in a plastic mixture of dodecenyl succinic anhydride, Araldite 6005, and Epon 812. Specific details of fixation and embedding are contained in a previous publication (14). Sections were cut on a Porter-Blum MT-2 microtome (Ivan Sorvall, Inc., Norwalk, Conn.) with a diamond knife and stained 5 to 60 sec with Reynolds' lead citrate (16).

Electron microscopy. Specimens were examined with an Hitachi HS-7S electron microscope with double condenser and 50 kv accelerating voltage. Initial magnifications ranged from 14,500 to 40,600. Micrographs were taken on contrast grade Kodak projector slides or on Kodak contrast process Ortho film.

RESULTS

Spore integuments. The early morphological events of sporulation in *C. cochlearium* 6B proceed, in most regards, according to sequences already described for sporeformers (2, 3, 7, 11, 13, 21). The spore septum forms near the pole of the cell enclosing a portion of the cell nuclear material. Further elaboration of this double membrane system results eventually in the completion of the forespore. The next prominent feature is deposition of spore coat structures which is initiated in some cases prior to the completion of the forespore (Fig. 1). The inner coat, laid down on the surface of the forespore outer membrane as a featureless structure, and the middle coat, first appearing as dense patches on the outer surface of the inner coat, appear simultaneously (Fig. 1) and precede cortex formation

(Fig. 2). The inner coat appears structurally complete at a time when the middle coat is still incompletely formed (Fig. 2). At this stage the first indication of a third coat structure (outer coat) is seen (Fig. 2).

This outer coat of the spore, first detected only when other morphological features are well developed (Fig. 2), exists in cross sections of mature sporangia in the form of curved segments (plates) arranged so as to form surface peaks or ridges (Fig. 3). The resulting starlike appearance is much more pronounced in sections of free spores (Fig. 4) and is apparent also as ridges in replicas of free spores (Fig. 5). The entire spore is enclosed in a delicate integument which is not, however, typically exosporiumlike in appearance (Fig. 4). This latter structure has not been detected in sections of sporangia or by carbon replication of free spores.

The outer spore coat (Fig. 4) is a complex structure consisting of seven discrete layers (Fig. 6) with a combined thickness of approximately 27 nm. The two delicate outer black layers measure approximately 2.6 nm each, the two underlying light layers measure approximately 3.5 nm each, the two thick inner dark layers measure 6.5 nm each, and the central light layer measures 2.2 nm.

Spore appendages. Longitudinal sections of sporangia reveal additional features. The oval spore is formed at the extreme pole of the cell (Fig. 7), which appears swollen at this site. As coat and cortex formation proceed, spore appendages and crystalline inclusions (15) appear in the vegetative cytoplasm (Fig. 8). When the spore is fully mature, numerous appendage structures are present in the sporangium as long, straight tubes, some of which extend to the distal end of the cell (Fig. 9).

Cross sections of sporgania at sites distal to the spore body reveal appendage structures embedded in the vegetative cytoplasm (Fig. 10 and 11). Each appendage consists of a central shaft region, which is considered to be tubular and measures approximately 68 nm in diameter (Fig. 10), and a surrounding zone (Fig. 10), which is designated as fibrillar (*see* Fig. 14). The overall diameter of the appendages in section (shaft plus fibrillar region) measures approximately 130 nm (Fig. 10). As many as nine such appendage structures are present in some sporangia (Fig. 11). An oblique section of a sporangium undergoing lysis suggests the tubular nature of the appendages (arrows, Fig. 12).

Carbon replicas of free spores show a rough spore surface and attached spore appendages quite variable in length (Fig. 13; longest appendage, 3.4 μm). The appendage smooth shaft region and the terminal fibrillar region are readily apparent in such preparations (Fig. 13). Negative stains establish that the individual appendage fibrils of this terminal region are approximately 50 nm in length, are approximately 4 nm in width, and are composed of spherical subunits (Fig. 14). Overall appendage width (shaft plus fibrillar region) by this technique (negative stain) is about 190 nm, and shaft width alone (flattened) is about 95 nm (Fig. 14). The tubular appendage shaft wall appears quite thin (Fig. 14) and exhibits ordered fine structure (Fig. 10 and 14).

The appendages appear to originate in the spore coat structures (Fig. 15 and 16).

DISCUSSION

The most striking feature of the spores of *C. cochlearium* 6B is, of course, the presence of appendages. It is now well established that some *Clostridium* spores have elaborate appendage structures, but their occurrence on spores of *C. cochlearium* has not been reported previously. The appendages of *C. cochlearium* 6B are attached to one end only of the ovoid spore (Fig. 13), as is the case in *C. taeniosporum* (9, 17). However, a specialized spore attachment structure (trunk) is absent (17). Certain other anaerobic spores have appendages extending from both spore ends (14), from the lateral surface of the spore body (20), or distributed randomly over the spore surface (5, 20). The structural continuity of *C. cochlearium* 6B appendages with, or origin in, the coat integuments seems clear enough (Fig. 15 and 16). This feature is shared by most, if not all, spore appendage types described to date.

The tubular nature of the spore appendages of *C. cochlearium* 6B is a property also of the spore appendages of *C. botulinum* type E (5), those of certain strains of *C. bifermentans* (6, 14, 20) and *C. sordellii* (6), and those of nontoxigenic mutants of *C. botulinum* type E (5). In addition, the tubular appendages of *C. cochlearium* 6B possess a fibrillar region similar in some respects to the fibrillar region of other spore appendage types (14, 20).

Whereas the spore appendages of *C. cochlearium* 6B have certain structural features in common with other spore appendage types, there are significant differences with respect to their disposition in sporangia and uniformity of length. The appendages of *C. cochlearium* 6B exist in the sporangium as tubes extending straight out into the cytoplasm without apparent coiling (Fig. 9), whereas the hirsute tubular appendages and the feather-like appendages of strains of *C. bifermentans* coil about in the sporangial cytoplasm in positions both internal and external to the exosporium (14, 20). In addition, the tubular

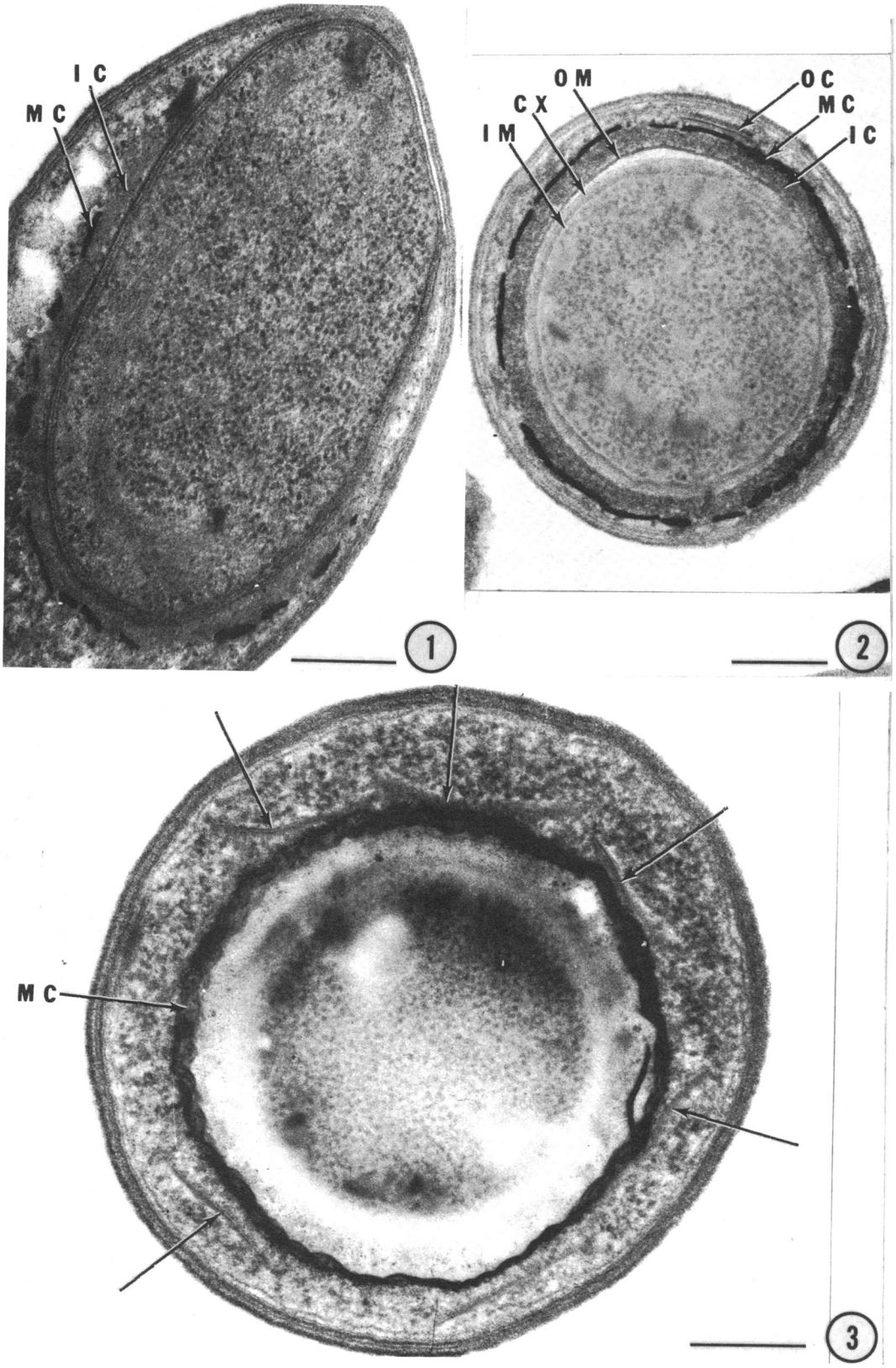

FIG. 1 to 3

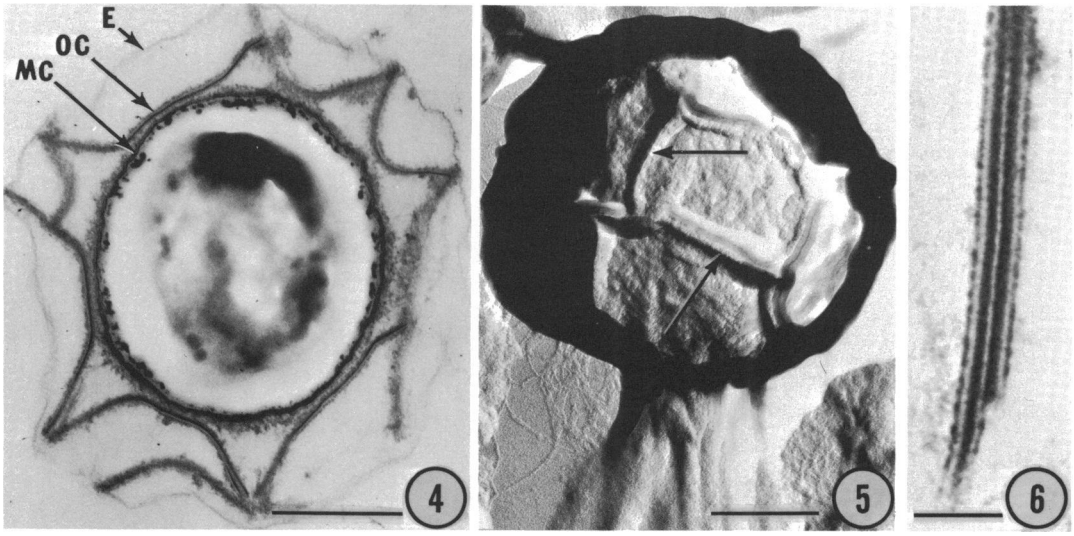

FIG. 4. *Free spore showing relationship between middle coat (MC) and outer coat (OC). A delicate structure, tentatively designated as an exosporium (E), encloses the spore. Bar indicates 0.3 μm.*

FIG. 5. *Free spore showing surface ridges (arrows) due to outer coat. Replica of spore fixed in 4% formaldehyde. Bar indicates 0.3 μm.*

FIG. 6. *Cross section of spore outer coat. Bar indicates 0.05 μm.*

appendages of *C. cochlearium* 6B are quite variable in length (Fig. 13), whereas those of *C. bifermentans* (14, 20) and those of *C. taeniosporum* (17) are considerably more uniform in this regard.

The outermost spore integument designated as outer coat (OC, Fig. 4) is a surprisingly complex multilayered structure (Fig. 6) which exhibits bilateral symmetry. The development of this outer coat lags significantly behind that of the other spore structural entities (Fig. 1–3). This integument is laid down in the form of plates (Fig. 3) whose edges curve outwards from the spore in such a manner that there exists a considerable cytoplasmic space between it and the middle coat (Fig. 3 and 4), particularly at the apices of the former (Fig. 4). This integument, in section, appears somewhat similar to the discontinuous laminated parasporal inclusion body of *Bacillus cereus* (4). Fine structure detail within the respective layers has not been observed.

The general pattern of the sporulation process described for other sporeformers (13, 21) appears applicable to *C. cochlearium* 6B with the exception that coat formation is fairly advanced (Fig. 1) before any evidence of cortex formation is noted (Fig. 2). This is in apparent contrast to *B. cereus* (21) and *B. coagulans* (13), in which initiation of cortex formation appears to be the earlier event.

The crystalline inclusions found in sporangia of *C. cochlearium* 6B (Fig. 8 and 9) have been considered in a previous report (15) and may result from defective phage production. This hypothesis is supported by the recent report of similar crystalline bodies formed in *B. thuringiensis* infected with bacteriophage (12).

The detection of spore appendages in *C. cochlearium* adds one more species to a growing list of organisms which produce such structures (6, 14, 20). Although numerous new species have been created with spore fine structure, the apparent primary criterion for speciation (9, 10), it is important to recognize that neither particular types of appendages, nor the presence or absence of appendages, are species-constant characters. Diversity of spore fine structure exists among strains of *C. botulinum* (6) and *C. bifermentans*

FIG. 1. *Sporangium section. Inner coat (IC) is laid down on the surface of the outer forespore membrane, and the middle coat (MC) appears as patches on the surface of the inner coat. Bar indicates 0.2 μm.*

FIG. 2. *Sporangium cross section showing the relationship between the inner coat (IC) and the outer membrane (OM), the first indication of an outer coat (OC) external to the almost complete middle coat (MC), and the cortex (CX) forming between the inner membrane (IM) and the outer membrane (OM). Bar indicates 0.2 μm.*

FIG. 3. *Cross section of sporangium with maturing spore. The outer coat is now present at multiple sites (arrows) external to the completed middle coat (MC). Bar indicates 0.2 μm.*

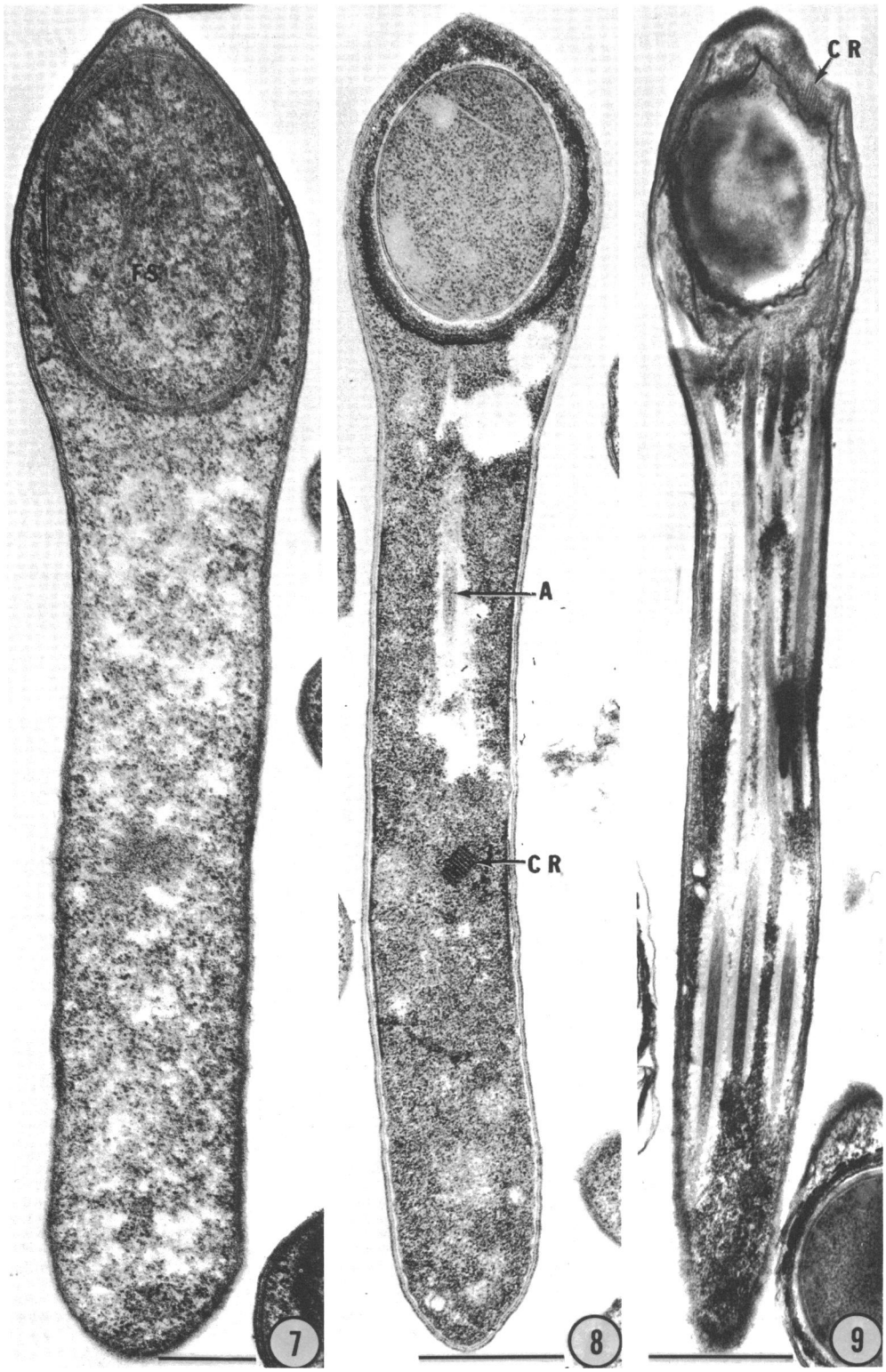

FIG. 7. *Longitudinal section of sporangium with completed forespore (FS). Bar indicates 0.2 μm.*

FIG. 8. *Longitudinal section of sporangium with an immature spore, an appendage (A), and a crystal inclusion (CR). Bar indicates 0.5 μm.*

FIG. 9. *Longitudinal section through sporangium with mature spore. Note a crystal (CR) near the spore and numerous long, straight appendages in the vegetative cytoplasm. Bar indicates 0.5 μm.*

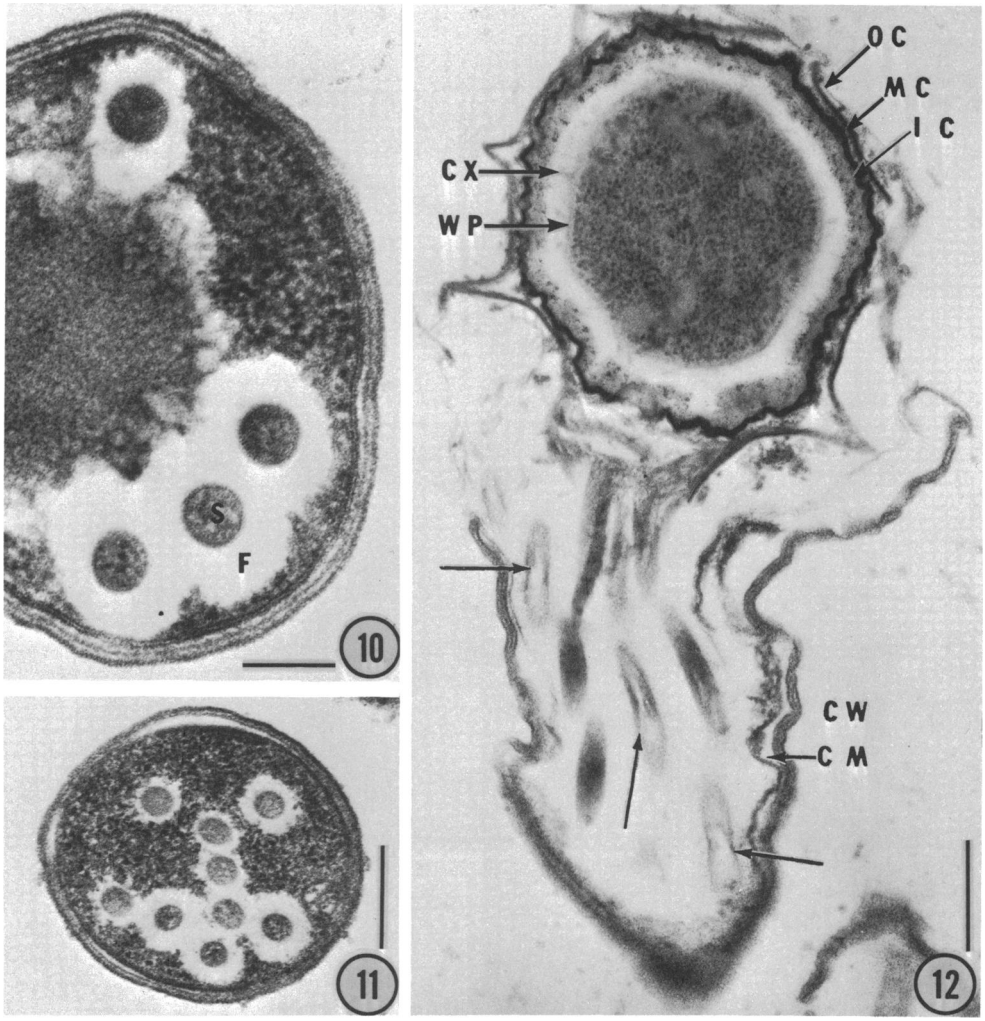

FIG. 10. *Sporangium showing the shaft (S) and fibrillar regions (F) of four spore appendages in cross section. Bar indicates 0.1 μm.*

FIG. 11. *Sporangium with nine spore appendages. Cross section. Bar indicates 0.2 μm.*

FIG. 12. *Oblique section of a lysing sporangium. Note the mature spore with its associated tubular appendages (arrows) and the spore outer coat (OC), middle coat (MC), inner coat (IC), cortex (CX), and wall primordium (WP) of the central body. Cell wall (CW). Cytoplasmic membrane (CM). Bar indicates 0.2 μm.*

(6, 14, 20), and preliminary data suggest that spore appendages do not occur in all strains of *C. cochlearium* (*unpublished data*).

Electron microscope studies should play a role in the establishment of sound morphological taxonomic criteria. This may be particularly applicable in the case of sporeformers where systematic morphological studies may reveal natural relationships not presently recognized. It would be premature to make specific taxonomic proposals until a great deal more information has been assembled; however, it is not too early to give recognition to the morphological diversity of spores within established species.

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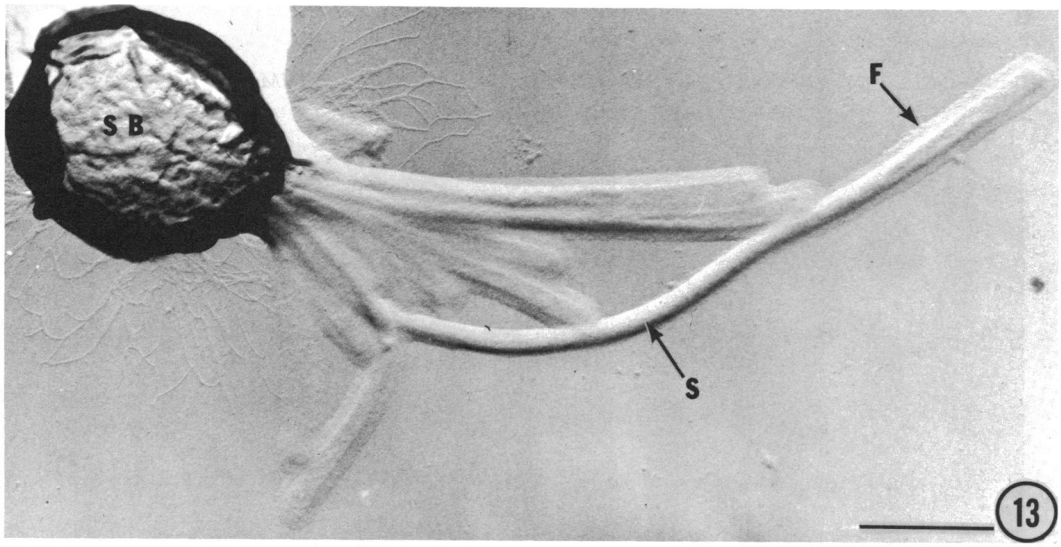

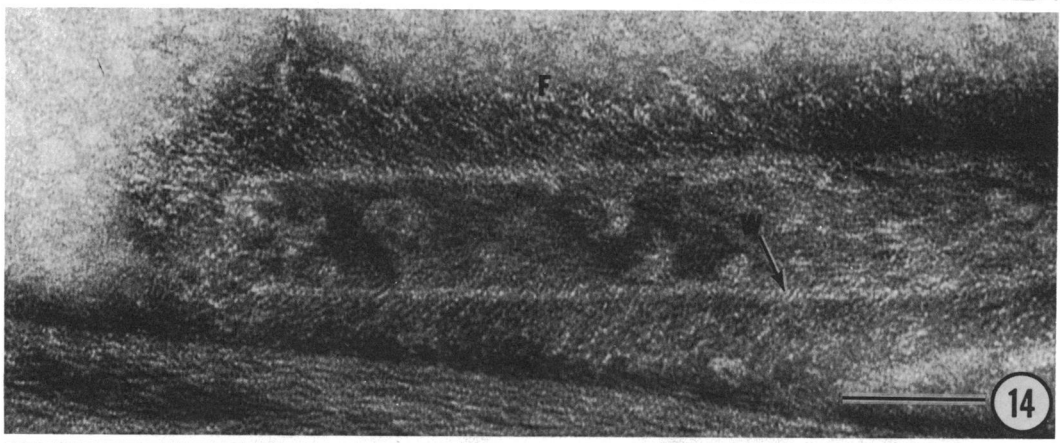

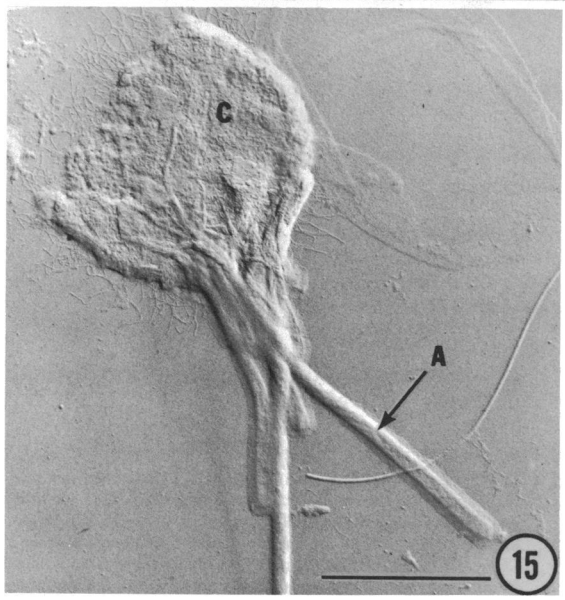

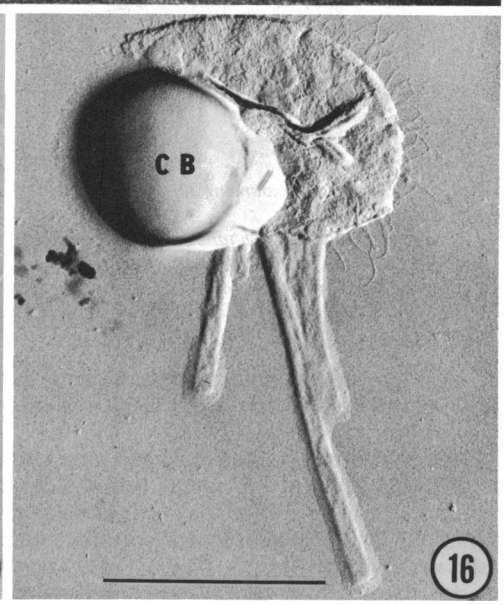

FIG. 13. *Free spore with numerous appendages attached to the spore body (SB). Each appendage consists of a tubular shaft region (S) and a terminal fibrillar region (F). Replica. Bar indicates 0.5 μm.*

FIG. 14. *Detail of an appendage showing the fibrillar (F) region surrounding the thin-walled (W) tubular shaft. Negative stain with 2% uranyl acetate, pH 4.3. Bar indicates 0.1 μm.*

FIG. 15. *Spore coat integument (C) with appendages (A) attached. Replica. Bar indicates 1 μm.*

FIG. 16. *Central body (CB) released from spore coat structures. Replica. Bar indicates 1 μm.*

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